Modulation of the immune system for treatment of atherosclerosis

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Vaccination with ApoB100 derived peptide p210 does not provide atheroprotection in LDLr deficient, hApoB$^{100/100}$ transgenic mice.

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Abstract

Several vaccination strategies using ApoB100-derived peptide p210 have been found to be capable of reducing atherosclerosis. The atheroprotective mechanism of p210 vaccination is however disputed, and has been dedicated to induction of p210 antibodies, regulatory B cells and CD4 T cells, and cytolytic CD8 T cells. In this study we aimed to induce tolerance towards p210 through oral delivery of p210 coupled to the cholera toxin B-subunit (CTB), and aimed to stimulate a humoral and cellular response against p210 through subcutaneous alum-adjuvanted vaccination of p210 coupled to Pan DR-epitope (PADRE), in LDLr deficient, $hApoB^{100/100}$ transgenic (HuBL) mice.

CTB-p210 and PADRE-p210 administrations induced p210 IgG, and CTB-p210 also induced IL-10 producing regulatory B cells. We did not observe a significant impact of either p210 treatment on the CD4 T cell and CD8 T cell populations previously reported to mediate the atheroprotective effects of p210. The MHC binding properties of p210 make it unlikely that p210 effectively induces p210 specific T cells, however enhanced cell death in CTB-p210 but not in CTB or p210 stimulated splenocyte cultures is suggestive of adjuvant properties of p210, which could explain the divergent effects reported of vaccination with p210.

We did not observe reduced atherosclerosis upon p210 treatment, however as p210 is part of the LDLr binding site in ApoB100, it is possible that p210 antibodies affect atherosclerosis through modulation of the interaction between ApoB100 and the LDLr, which effect would have been missed with the LDLr deficient model used in this study. Alternatively, adjuvant properties of p210 could have induced atheroprotection in previous studies.

Introduction

Cardiovascular disease is still the primary cause of death in the world *(1)*. Atherosclerosis is the dominant underlying pathology of cardiovascular deaths. The pathogenesis of atherosclerosis is hallmarked by retention and accumulation of cholesterol rich LDL in the vessel wall of middle to large sized arteries. In the arterial wall LDL undergoes oxidative modifications, forming oxLDL, which triggers a chronic local immune response. Early atherosclerosis is dominated by the influx of monocytes in the atherosclerotic lesions which differentiate to macrophages and take up large quantities of oxLDL *(2)*. Presentation of LDL by dendritic cells and macrophages (antigen presenting cells, APCs) induces humoral and cellular adaptive immune responses to LDL, resulting in a pathogenic Th1 skewed immune response *(3, 4)*. Classically treatment of atherosclerosis has been focused on lowering lipid levels. This has been effective, however many patients carry a residual risk to cardiovascular events due to unresolved inflammation, even after successful lipid lowering *(5)*. Administration of neutralizing antibodies against IL-1β in the CANTOS trial reduced major cardiovascular events by up to 15% in the higher dose groups *(6, 7)*, supporting the rationale of reducing inflammation for treatment of atherosclerosis.

Since LDL has been identified as a major auto-antigen in atherosclerosis, multiple research groups have modulated the immune response towards LDL in preclinical models, successfully reducing atherosclerosis *(8)*. The clinical application of apoB100, the main protein in LDL, is hampered because of its large size and heterogeneous nature, and researchers have attempted to identify immunogenic epitopes in LDL for clinical purposes. The 20 amino acid long p210 peptide is one of the LDL ApoB100 derived antigens that in multiple different formulations and vaccination strategies, has proven to be capable of reducing atherosclerosis *(9–12)*. The protective mechanism(s) by which p210 vaccination induces atheroprotection are however still disputed *(8)*, which may hamper the clinical use of p210 vaccination. The p210 peptide was originally described as an antigen that is recognized by antibodies in serum of cardiovascular patients *(13)*, identifying p210 as an antibody epitope. In line with this, the atheroprotective effects of p210 have been attributed to induction of p210 specific antibodies *(12)*. However other papers have dedicated the atheroprotective properties of p210, utilizing several different formulations, administration routes and administration schedules, to induction of Bregs *(10)*, CD4 Tregs *(9, 10)*, and cytotoxic CD8 T cells *(11, 14)*.

In this study we aimed to further delve into the effects of p210 vaccination on the immune system and its atheroprotective effects. Since it is generally accepted that atheroprotection can be accomplished by induction of a regulatory response towards intact (ox)LDL *(15)* or parts of LDL *(16, 17)*, we first aimed to induce a tolerogenic response towards p210 by oral administration of p210 coupled to cholera toxin B (CTB). Repeated low dose antigen administration via the oral route is known to induce regulatory antigen specific adaptive immune cells, inducing so called oral tolerance *(18)*. CTB can induce tolerogenic dendritic

cells *(19)* and B cells *(10, 20)*, and enhances mucosal delivery of coupled antigens through interaction with GM1 gangliosides, greatly enhancing antigen specific regulatory CD4 T cell numbers in vivo *(21)*. To investigate the atheroprotective potential of p210 antibodies and p210 specific conventional effector T cells, we intraperitoneally vaccinated mice with p210 coupled to PADRE, adjuvanted with alum.

Since p210 is not completely homologous to the corresponding murine sequence in ApoB100 (90% homology, accession NP_033823.2 vs p210), we aimed to use a preclinical model with endogenous expression of human ApoB100, which could impact thymic selection of T cell clones specific for human apoB100 and p210, and in vivo presentation of the cognate antigen for specific T cell clones. Furthermore in all published studies regarding p210 immunization in atherosclerosis ApoE deficient mice were utilized. Since p210 is part of the LDL receptor binding site A in ApoB100 *(22, 23)*, we wondered whether the protective effect of p210 vaccination still was observed in the absence of LDLr. Therefore we used LDLr deficient and human $ApoB^{100/100}$ transgenic (HuBL) mice *(23, 24)* in the experiments described in this paper.

Results

Oral vaccination with CTB-p210 induces Bregs, and p210-specific IgG and IgA

To establish a regulatory response towards p210, female HuBL mice received an oral gavage with CTB-p210 (30 µg) every other day in the first week (4 times), after which p210-CTB was orally administered weekly to maintain a p210 specific immune response. In parallel to CTB-p210 administration, the control group orally received CTB (same molar amount). From the start of treatment animals were fed a western type diet to induce atherosclerosis, until sacrifice 8 weeks later. Since previously CTB-p210 was found to induce Bregs in vitro *(10)*, we assessed the splenic B cell population for IL-10^+ Bregs (B10 cells) with flow cytometric analysis of splenocyte cultures incubated with PMA/ionomycin and Monensin for 5h (Fig. 1B). The overall levels of splenic B cells (prior to culture) did not differ between CTB and CTB-p210 treated mice (Fig. 1A), but in line with the in vitro data of Rattik CTB-p210 treatment increased B10 cell levels compared to CTB treatment (Fig. 1C). Because p210 was initially identified as an antibody epitope *(13)*, we assessed the effect of oral CTB-p210 vaccination on the B cell population and p210 specific antibody levels. Oral CTB-p210 administration increased IgA and IgG levels, but not IgM levels, against native p210 (Fig. 1D) and malondialdehyde (MDA) modified p210 (Fig. 1E) compared to CTB administration.

Oral CTB-p210 administration does not induce Tregs

Because nasal CTB-p210 administration was previously found to induce CD4 Tregs in vivo *(9)* and CTB-p210 pulsed B cells induced CD4 Tregs in vitro *(10)*, we assessed Treg levels in several lymphoid organs. We did not observe enhanced levels of $FoxP3^+CD25^+$ CD4 T cells (Fig. 2A) and $FoxP3^+CD25^+$ CD8 T cells (Fig. 2C) in freshly isolated splenocytes, or enhanced levels of IL-10^+ CD4 T cells (Fig. 2B) and IL-10^+ CD8 T cells (Fig. 2D) in PMA/ionomycin stimulated

splenocyte cultures of P210-CTB treated mice. Since after oral administration of CTB-OVA the highest levels of Tregs were measured in the Peyer's patches and mesenteric lymph nodes draining the intestines *(25)*, we also assessed FoxP3^{+}CD25$^{+/-}$ CD4 T cell levels in Peyer's Patches (Fig. S1A) and mesenteric lymph nodes (Fig. S1B), and atherosclerosis relevant mediastinal lymph nodes (Fig. S1D), draining the aortic arch, and in circulation (Fig. S1C). In neither of the assessed organs we observed enhanced Treg levels, suggesting that oral CTB-p210 administration did not enhance Treg levels over oral CTB administration. Moreover, we did not observe a difference in IFN-γ^{+} CD4 T cells between CTB and CTB-p210 treated (Fig. 2B), indicating that the atherogenic Th1 response was not significantly altered by CTB-p210 compared to CTB administration.

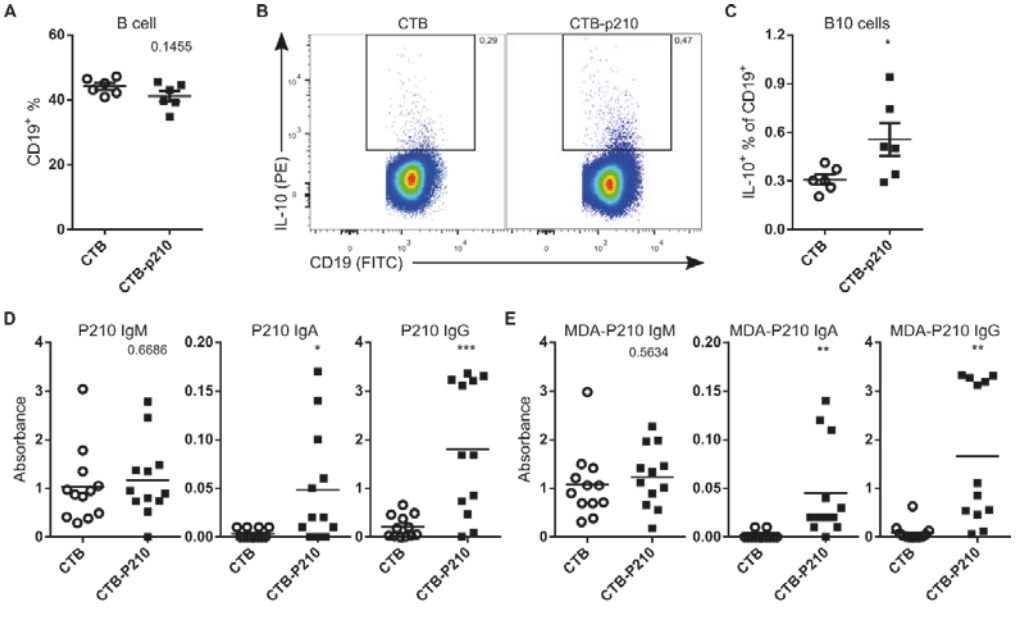

Fig. 1 ***Oral CTB-p210 administration induces B10 cells and anti-p210 IgA and IgG antibodies. A****) Quantification of B cell content in spleen as assessed by flow cytometry.* ***B****) Representative flow cytometry plots of the CD19^{+}CD4^{-}CD8^{-} population of splenocytes cultures incubated with PMA/IONO, and Brefeldin A and Monensin for 5h.* ***C****) Quantification of IL-10^{+} cells in the B cell population of PMA/IONO stimulated splenocytes cultures. Quantification of ELISA measurements of IgM, IgA, and IgG antibodies against* ***D****) native p210, and* ***E****) malondialdehyde (MDA)-modified p210, in blood serum (1:50 dilution) obtained at sacrifice. Represented as mean ± SEM, unpaired two-tailed T test, * p < 0.05, ** p < 0.01, *** p < 0.001.*

Oral CTB-p210 administration induces splenocyte reactivity towards oxLDL

To assess whether vaccination with CTB-p210 had affected the immune reactivity towards oxLDL, we subjected splenocytes to a thymidine incorporation assay. Surprisingly, both basal proliferation (Fig. 3A) and oxLDL induced proliferation (Fig. 3B) were increased in splenocyte cultures from CTB-p210 treated mice compared to splenocyte cultures from CTB treated mice. Furthermore overall thymidine incorporation was similar in αCD3/αCD28 cultures from both treatment groups (Fig. 2C), indicating that overall proliferative capacity of splenic T cells was similar in CTB-p210 and CTB treated mice. In an effort to quantify CTB-p210 treatment relevant antigen specific T cell responses, we exposed splenocytes from both treatment groups to freshly isolated LDL (5 µg/ml), copper oxidized oxLDL (5 µg/ml), p210 (2 µM), CTB (2 µM), or CTB-p210 (2 µM) for 5h after which levels of IL-2, IL-10 and IFN-γ producing CD4 and CD8 T cells were assessed by flow cytometry. We did not observe significant changes in cytokine production between the treatment groups (data not shown), however we did observe reduced cell viability in splenocytes cultured with CTB-p210 irrespective of in vivo treatment (Fig. 3DE).

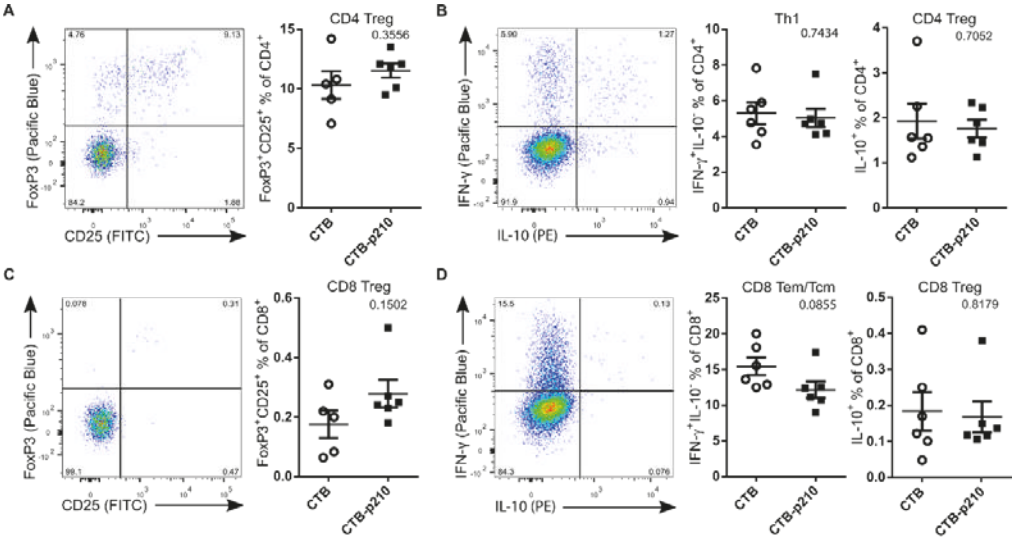

Fig. 2 ***Splenic regulatory T cell and Th1 cell levels are not affected by oral CTB-p210 administration.*** ***A****) Representative flow cytometry plots of the gating of regulatory CD25⁺FoxP3⁺ cells and quantification in the splenic CD4 T cell population and* ***C****) CD8 T cell population.* ***B****) 2*10⁶ splenocytes per well were incubated with PMA, ionomycin, Monensin and Brefeldin A for 5h after which IFN-γ⁺ and IL-10⁺ CD4 T cells and* ***D****) CD8 T cells were flow cytometrically assessed and quantified. Represented as mean ± SEM,*

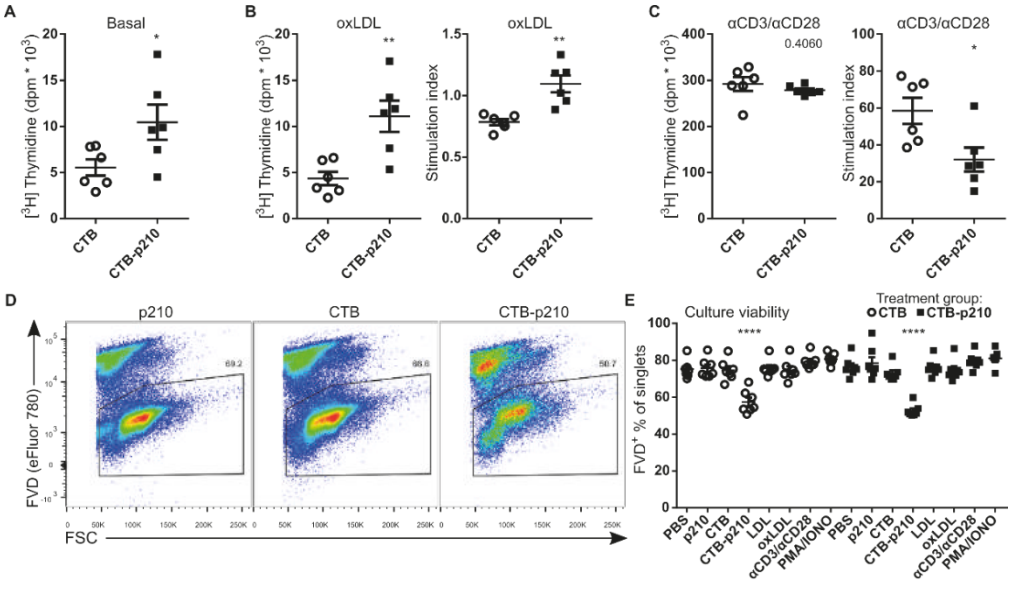

Fig. 3 ***CTB-p210 induces oxLDL reactivity in ex vivo splenocyte cultures and causes cell death in vitro.*** ***A****) Basal splenocyte proliferation* ***B****) oxLDL (5 µg/ml) induced proliferation and* ***C****) αCD3/αCD28 induced T cell proliferation as assessed by a thymidine incorporation assay in splenocyte cultures from CTB and CTB-p210 treated mice.* ***D****) Representative flow cytometry plots of the singlet population of splenocyte cultures (2*10^6 cells/well) from the same CTB treated mice, incubated for 5 hours with p210 (2 µM), CTB (2 µM), or CTB-p210 (2 µM), and gating for viable cells.* ***E****) Quantification of culture viability of splenocyte cultures from CTB and CTB-p210 treated mice, incubated for 5 hours with PBS, p210 (2 µM), CTB(2 µM), CTB-p210(2 µM), LDL (5 µg/ml), oxLDL (5 µg/ml), αCD3/αCD28, or PMA+Ionomycin, and Brefeldin A and Monensin, as assessed with flow cytometry. Represented as mean ± SEM,* ***A-C****) unpaired two-tailed T test,* ***E)*** *One-Way ANOVA with Holm-Šídák posttest, * p < 0.05, ** p < 0.01, **** p < 0.0001.*

Antibodies against p210 are not protective in LDLr deficient hApoB100tg mice.

To assess the effect of CTB-p210 vaccination on atherogenesis we histologically assessed the atherosclerotic lesion size in the aortic root (Fig. 4A) and brachiocephalic artery (Fig. 4C). The average aortic root lesion size was not different between the CTB treated group (636667 ± 170356 µm^2) and the CTB-p210 treated group (693083 ± 135938 µm^2) (Fig. 4B). Similarly brachiocephalic average lesion size and lesion volume did not differ between CTB (avg. lesion: 139790 ± 73282 µm^2; volume: 1.48*10^8 ± 1.29*10^8 µm^2) and CTB-p210 treated (avg. lesion: 94512 ± 69641 µm^2; volume: 9.46*10^7 ± 8.46*10^{7}µm^2) (Fig. 4D). Moreover, there was no correlation between the atherosclerotic lesion size in the aortic root and IgM, IgA, and IgG levels against native p210 (Fig. S2A) and MDA-modified p210 (Fig. S2B).

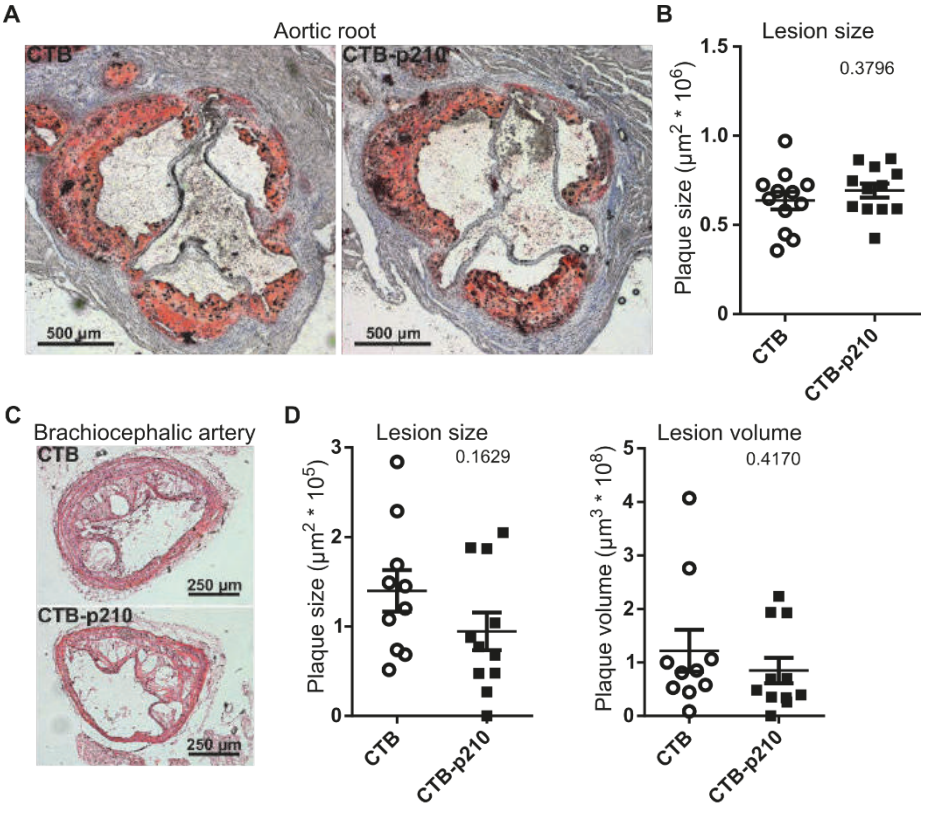

Fig. 4 ***Oral CTB-p210 vaccination does not reduce atherosclerotic plaque size in HuBL mice. A****) Transverse sections of the aortic root stained with oil red o* ***B****) and quantification of the average lesion size in the aortic root.* ***C****) Sections of the brachiocephalic artery stained with H&E and* ***D****) quantification of the average lesion size and lesion volume in the brachiocephalic artery. Represented as mean ± SEM, unpaired two-tailed T test.*

The only p210 specific response we could detect after oral CTB-p210 administration was the induction of p210 specific antibodies. To ensure that we did not underestimate the atheroprotective effect of p210 antibodies, possible due to induction of insufficient levels of p210 antibodies to significantly impact atherogenesis, we performed an additional experiment. In this experiment we subcutaneously immunized HuBL mice with p210 coupled to PADRE (Pan-DR-epitope), a strong MHC-II epitope which promotes T cell dependent antigen production *(26)*, and used alum as an adjuvant to stimulate a humoral response *(27)* and a conventional effector CD4 and CD8 T cell response against p210. Since a p210 specific atheroprotective CD8 T cell response was reported after alum adjuvanted vaccination with cBSA-p210, we were also cautious of a possible CD8 T cell response induced by our vaccination scheme.

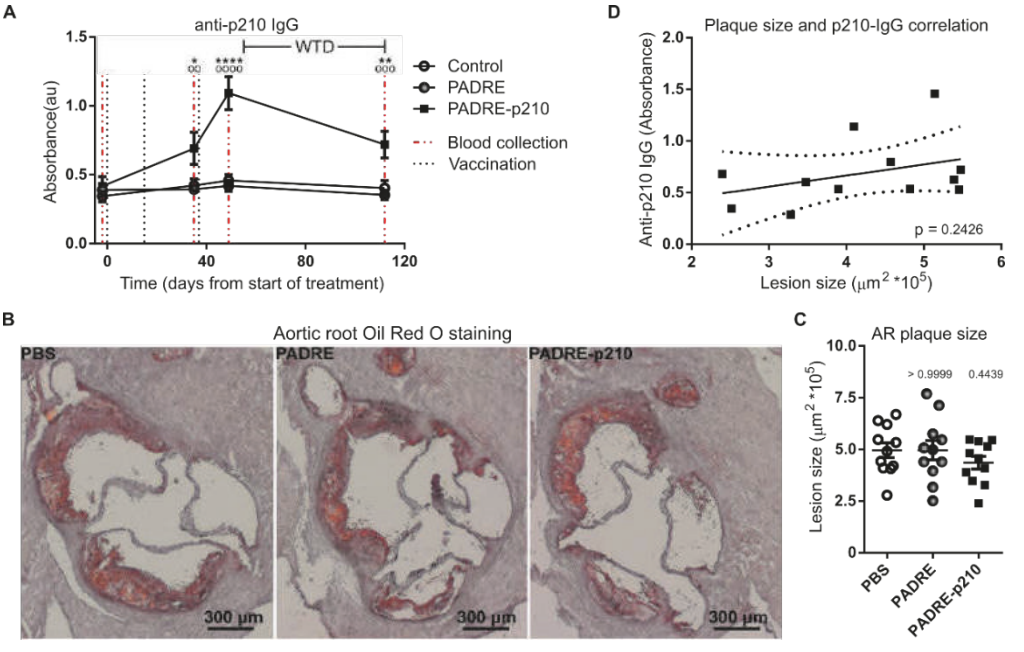

Fig. 5 ***PADRE-p210 vaccination induces high p210 specific IgG levels but does not act atheroprotective in HuBL mice.*** ***A****) Experimental setup and p210 IgG levels blood plasma (1:1000 dilution).* ***B****) Representative transverse sections of the aortic root stained with ORO,* ***C****) used for the quantification of the average atherosclerotic lesion size in the aortic root (AR).* ***D****) Correlation analysis of p210 antibody levels in blood serum obtained at sacrifice from PADRE-p210 treated animals, and the average atherosclerotic lesion size in the atherosclerotic root. Represented as mean ± SEM,* ***A****) Two-Way ANOVA with Holm-Šídák posttest, significance of control vs PADRE-p210 treatment depicted by (*) and of PADRE vs PADRE p210 by (°), * p < 0.05, **/°° p < 0.01, °°° p < 0.001 ****/°°°° p < 0.0001.* ***C****) One-Way ANOVA with Holm-Šídák posttest.* ***D****) Scatter dot plot depicted with trend line (solid line), 95% Confidence interval (dotted lines).*

Mice were subcutaneously vaccinated 3 times in 37 days with PADRE-p210 (25 µg, 6.7 nmol) in alum, PADRE (9.1 µg, 6.7 nmol) in alum, or PBS (Fig. 5A). Increased levels of p210 IgG antibodies were observed in the p210-PADRE treated group compared to PADRE and PBS treated groups after the second immunization and were further boosted with a third vaccination, after which mice received WTD until sacrifice (Fig. 5A). We did not detect differences between treatment groups in levels of CD8 T cells, $Ki67^+$ proliferating CD8 T cells, $CD44^+$ antigen experienced CD8 T cells, and $FoxP3^+$ CD8 Tregs in mediastinal lymph nodes (data not shown) and spleen (Fig. S3A). The effect of PADRE-p210 on atherosclerosis was assessed by histological analysis of aortic root tissue sections (Fig. 5B) p210-PADRE treatment did not affect aortic root lesion size (Fig. 5C) (PBS: 490517 ± 118811 μm^2 PADRE: 495962 ± 154416 μm^2 PADRE-p210: 436183 ± 102236 μm^2). Anti-p210 IgG levels of PADRE-p210 treated animals did not correlate with plaque size (Fig. 5D), similar to what we observed in CTB-p210 treated mice.

Discussion

Cardiovascular disease is the primary cause of death in the world with atherosclerosis as its main underlying pathology *(1)*. Atherosclerosis is driven by dyslipidemia and inflammation. The main focus of atherosclerosis treatment has classically been the normalization of circulating lipid levels. Normalizing lipid levels in circulation has reduced cardiovascular events by reducing the deposition and accumulation of cholesterol rich LDL in the artery wall *(28, 29)*. Furthermore normalization of lipid levels also reduces inflammation, however in many CVD patients in which treatment of dyslipidemia is successful, inflammation is not completely resolved and poses a residual risk for a CV event *(5)*. Recently, results from the CANTOS trial, in which a human IL-1β neutralizing antibody was used to dampen inflammation, provided proof that inhibition of inflammation is clinically effective in human, reducing major cardiovascular events by 15% *(7)*.

Preclinical studies have shown that inflammation in context of atherosclerosis can also be inhibited through modulation of the adaptive response to plaque constituents, including HSPs *(30, 31)* , complete (ox)LDL *(15)* and parts of (ox)LDL *(32)*, which lead to a reduction in atherosclerosis. Because LDL is heterogeneous containing lipids and a single, large apolipoprotein B-100, research groups have put effort into identifying immunogenic epitopes in the ApoB100 protein to use for modulating the immune response against LDL *(13, 16, 33)*. One of the epitopes that was identified by a peptide library screen for antibody binding derived from human pooled blood is p210 *(13)*. Since then, multiple immunization studies with p210 have been performed resulting in reduced atherosclerosis in ApoE deficient mice, although the mechanism of action is still disputed *(34, 35)*. To obtain new insights into the immunological mechanism of action of p210 we aimed to assess the effect of p210 vaccination on atherosclerosis and the immune system in an LDL receptor deficient mouse model with endogenous expression of human ApoB100, the HuBL mouse model.

Because p210 mediated atheroprotection has been suggested to originate from the induction of regulatory CD4 T cells *(9)*, regulatory B cells *(10)*, conventional effector CD8 T cells *(11, 14)*, and p210 specific antibodies *(12)* depending on formulation and mode of administration, we employed two vaccination strategies to promote induction of adaptive responses against p210. On the one hand, we opted to deliver p210 fused to the tolerogenic adjuvant CTB *(21)* and administered via the oral route to induce oral tolerance, which is known to induce regulatory B cells, and antigen-specific Tregs *(18)*. On the other hand, p210 was coupled to PADRE, adjuvanted with alum and injected subcutaneously to maximize p210 specific antibody production and induce conventional T cell responses.

Both vaccination approaches used in our study resulted in the induction of p210 specific antibodies in the treatment groups containing p210, indicating that both protocols broke tolerance towards a part of the endogenous protein apoB100. We did not observe an

atheroprotective effect of induction of antibodies against p210. In a recent study however, immunization with p210, and administration of antibodies against MDA-modified p210 resulted in reduced atherosclerosis due to p210-antibody mediated altered lipid handling and reduced inflammation *(12)*. Similarly, atheroprotection was observed in nasal CTB-p210 treated mice, inducing anti-p210 antibodies and CD4 Tregs *(9)*. The discrepancy in the observed atheroprotective effect of p210 immunization between the studies of Klingenberg and Zeng, and our study can be the result of the use of ApoE deficient mice in the previous studies, and the use of LDLr deficient animals in our study since p210 is part of LDLr binding site A in ApoB100 *(22, 23)*. In line with a role for the LDLr in the atheroprotective effects of p210 antibodies, LDLr mediated uptake of LDL by adipocytes was inhibited in vitro by p210 antibodies *(36)*. In macrophage cultures incubated with oxLDL, addition of p210 antibodies inhibited the formation of foam cells *(36)* and upregulated the expression of cholesterol efflux genes *(12)*, beneficial in the context of atherosclerosis. Induction of p210 and LDL cross-reactive antibodies with a p210 mimotope, was found to prevent high fat diet induced weight gain and liver steatosis in wild type C57BL/6 mice *(36)*, showing that anti-p210 antibodies can also improve lipid handling in vivo in WT animals. Through immunization with a p210 mimotope in the study of Kim, a peptide with a different amino acid sequence than p210 but capable of inducing antibodies cross reactive with p210 and LDL, the possibility that p210 specific T cells were induced and affected lipid handling was excluded. Moreover in humans, anti p210 IgM and IgG levels were correlated with improved carotid intima-media thickness parameters *(37)*, indicating atheroprotective properties of p210 antibodies in human. Interestingly the inverse correlations between anti-p210 IgG levels and baseline composite measures of carotid intima-media thickness disappeared when adjusted for known risk factors *(37)*, suggesting that improved lipid handling induced by anti-p210 antibodies *(12, 36)* might also occur in human.

Besides induction of protective antibodies, the protective effect of vaccination with p210 formulations has been dedicated to the induction of atheroprotective T cell populations *(9, 11, 14)*. However, after neither of the p210 immunizations we detected alterations in CD4 T cell or CD8 T cell populations, nor did we detect an atheroprotective effect which, if T cell dependent, should not have been affected by the use of an $LDLr^{-/-}$ or $ApoE^{-/-}$ model. Furthermore, immunomodulation using ApoB100 derived CD4 T cell epitopes was shown to be capable of reducing atherosclerosis in $ApoE^{-/-}$ mice *(16)* and $LDLr^{-/-}$ mice *(17)* before. It would be interesting to assess whether this favorable immunomodulation can still be achieved when commenced at later stages of atherosclerosis development, when immune tolerance towards plaque antigens like ApoB100 might have been eroded *(38)*. Lack of robust demonstration of p210 specific T cells is in line with in silico prediction tools of MHC binding which indicate that it is very unlikely that p210 itself or p210 derived peptides bind murine MHC-II (IAb) *(35)*, or MHC-I (H-2Kb and H-2Db) (Supplementary Table 1). This suggests that the previously observed T cell responses after p210 vaccination are likely dependent on an indirect mechanism. Similarly we observed enhanced cell death in splenocyte cultures

incubated with CTB-p210 irrespective of the treatment group, which was unlikely the result of a cytotoxic T cell response or regulatory T cell response towards CTB-p210, because flow cytometric analysis of the cultures did not reveal enhanced IFN-γ^+ or IL10$^+$ CD4 T cells or CD8 T cells, nor did p210 or CTB induce enhanced cell death. It is therefore conceivable that p210 potentiated the known pro-apoptotic properties of CTB *(39)*.

Adjuvant properties of p210 could explain the divergent immunological effects which have been described upon administration of different p210 formulations. As a matter of fact, the heparan sulfate proteoglycan and LDLr binding properties of the LDLr binding sites of ApoB100 were previously utilized to promote the uptake of the SIINFEKL peptide, promoting cross priming of CD8 T cells *(22)*. Furthermore, an enhanced FITC signal was detected in DCs cultured with p210-FITC but not in DCs incubated with FITC alone *(11)*, strongly suggestive that p210 can promote uptake of coupled proteins via heparan sulfate proteoglycans and the LDLr. Enhanced p210 mediated uptake and cross-presentation of cBSA, containing T cell epitopes, could therefore explain the observed induction of CD8 T cell activity in CTB-p210 vaccinated mice *(11)*. Due to the 70% homology between bovine albumin and murine albumin (accession M73993.1 vs accession BC049971.1), enhanced cBSA presentation is likely to cause induction of alloreactive and auto-reactive CD4 and CD8 T cell clones, and cross-reactive antibodies against murine albumin which is present in large quantities in atherosclerotic plaques *(40)*. Since immunization with cBSA reduces atherosclerosis *(41)*, the proposed adjuvant properties of p210 could therefore explain the atheroprotective effect of cBSA-p210 vaccination. Similarly, enhanced p210 mediated uptake of CTB, enhancing its tolerogenic activity, could also explain the enhanced induction of regulatory TGF-β^+ B cells in vitro with CTB-p210 compared to CTB-OVA *(10)* and the enhanced regulatory B10 cells that we observed in spleens of CTB-p210 treated mice.

In our immunization studies with CTB-p210 and PADRE-p210 we did not observe any improvement in plaque parameters after vaccination with p210. We did not observe previously reported atheroprotective CD4 and CD8 T cell responses, likely due to absence of T cell epitopes in p210. We did observe induction of anti-p210 antibodies. Failure of anti-p210 antibodies to modulate atherosclerosis in LDLr deficient HuBL mice used in this study, support the notion that anti-p210 antibodies act through modulation of the interaction of LDL with the LDLr. A conceivable explanation for the divergent immunological effects of vaccination with p210 which have been reported, are the strong indications that p210 possesses adjuvant properties and could thereby promote the activity of p210-coupled adjuvants and promote immune reactions against p210-coupled antigens.

Materials and methods

Animals

All animal work was approved by the Leiden University Animal Ethics Committee and the animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Breeding pairs from human ApoB100 transgenic LDLr$^{-/-}$ (HuBl) mice *(23, 24)* were a kind gift from prof. Jan Nilsson (Lund University), and further bred in house. HuBl mice were housed in individual ventilated cages with aspen bedding, in groups of 2–4 mice. Prior to inclusion of mice in experiments, expression of human ApoB100 was confirmed in blood plasma with a human ApoB100 ELISA (Mabtech). Mice were fed a regular chow diet prior to initiation of the *in vivo* experiments after which they were fed a Western-type diet (WTD) containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Purified p210 (human apoB-100 amino acids 3136 to 3155; KTTKQSFDLSVKAQYKKNKH) *(13)*, p210 gene fusion proteins with CTB and PADRE, and CTB and PADRE, derived from cytoplasmic inclusion bodies in E. coli like previously described *(42)*, were a kind gift from dr. Lebens, (University of Gothenburg), and administered as indicated in the results section. At the end-point of the studies, mice were anesthetized by subcutaneous injection with a mix of ketamine (100 mg/mL), sedazine (25 mg/mL) and atropine (0.5 mg/mL), retro-orbitally exsanguinated, and perfused with PBS.

In vitro assays

Basal splenocyte proliferation and proliferation in response to oxLDL (5ug/ml) or anti-CD3e (1 µg/mL) and anti-CD28 (0.5 µg/mL) (both from Thermo Fischer) were assessed by a ^{3}H-thymidine incorporation assay. Splenocytes (2*10^{5} cells/well) were cultured for 72h in RPMI (Lonza; supplemented with 10% FCS (GE Life Sciences), pen/strep Lonza) with mentioned stimuli or PBS (control) and incubated with 0.5 µCi/well ^{3}H-thymidine (Perkin Elmer) for the last 16 h. Cells were thoroughly washed with PBS and thereafter lysed with natriumhydroxide and taken up in Emulsifier-Safe™ (Perkin Elmer). ^{3}H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). Responses are expressed as the mean disintegrations per minute (dpm). The stimulation index (s.i.) was defined by dividing the dpm of stimulated conditions by the dpm of the PBS condition per mouse. Moreover, responsiveness of splenic CD4 T cells, CD8 T cells, and B cells, towards p210 (2 µM), CTB(2 µM), CTB-p210(2 µM), LDL (5 µg/ml), oxLDL (5 µg/ml), αCD3 (1 µg/mL)/αCD28(0.5 µg/mL), or PMA+Ionomycin, was assessed by incubation of splenocytes (2*10^{6} cell /well) with the indicated stimuli or PBS and incubation with Monensin and Brefeldin (Biolegend) for 5 hours, after which splenocyte cultures were assessed with flow cytometry.

Flow Cytometry

Extracellular staining (ECS) of single cell suspensions was performed in PBS with 2% FCS and αCD16/32 antibody (93, Biolegend) and eBioscience™ Fixable Viability Dye eFluor™ 780

(ThermoFisher) to discriminate between living and dead cells for 30 minutes at 4°C. For intracellular transcription factor staining after ECS, cells were fixed and permeabilized with the FoxP3 transcription factor buffer set (Thermofisher/eBioscience) according to manufacturer's instructions, and incubated with flow cytometry antibodies for 45 minutes at 4°C. Spleen and lymph nodes were mashed over a 70 µm cell strainer (Greiner) to obtain single cell suspensions and red blood cells were lysed with ACK lysis buffer if necessary.

The following antibodies were purchased at BD biosciences: The following antibodies were purchases at BD Biosciences: CD4 PerCP and Pacific Blue (RM4-5), CD44 PE/Cy7 (IM7) Biolegend: CD8 BV510 and APC (53-6.7); eBioscience/Thermofisher scientific: CD19 Pacific Blue and FITC (eBio1D3), CD19 PE/Cy7 (eBio1D3), CD25 APC (PC61.5), CD25 FITC (PC61.5), CD8-APC (16-10A1), FoxP3-PE (NRRF-30), IFN-γ (XMG1.2), IL-2 APC (JES6-5H4), IL-10 PE (JES5-16E3), Ki67 FITC (SolA15). Compensation measurements were performed using UltraComp eBeads (ThermoFisher) and ArC Amine-Reactive Compensation Beads (ThermoFisher). Cells were measured with a FACSCanto II (BD Biosciences) and a Cytoflex S flow cytometer (Beckman Coulter) and analyzed using FlowJo software (Tree Star, inc.).

P210 autoantibody determination

Native p210 and MDA-modified p210 (MDA-p210) antibodies were determined with an ELISA approach. MDA-p210 was obtained through incubation of p210 with 0.5M MDA for 3h at 37°C, and dialysis with PBS containing 1mM EDTA. High protein binding plates (Corning Costar) were coated with native p210 (20ug/ml) and MDA-p210 (20ug/ml) in PBS overnight at 4°C. Coated plates were washed with 0.01% Tween-20 (PBS-T) and blocked with PBS-T with 1% BSA for 1-2h at RT. Thereafter plates were incubated with plasma diluted 50 times with PBS-T (CTB-p210 study), or 1000 times with TBS-T + 1% BSA (PADRE-p210 study) for 2h at RT. Thereafter plates were incubated with secondary HRP conjugated Goat anti mouse IgM, IgA, and IgG antibodies (Thermo Fisher Scientific) for 1-2h at RT. A color reaction was developed with TMB substrate solution (Thermo Fisher Scientific), which was stopped with sulfuric acid (0.16M) and measured at 450nm using a spectrophotometer (Powerwave 340, Biotek).

Histology

Hearts were transversally cut in half and incubated in OCT medium for 30 minutes. After 30 minutes hearts were fast frozen on dry ice, and stored at -80°C until cryosections (10 µm) of the aortic root were made on collected on Superfrost Plus™ Adhesion Microscope Slides (ThermoFisher) and analyzed at 70 µm intervals. Neutral fats were stained with Oil Red O to determine the average lesion size of five subsequent sections of the aortic root containing 3 valvular leaflets with largest plaque area, as a measure of atherosclerotic lesion size.

Murine MHC-I binding prediction

The immune epitope database and analysis resource (iedb.org) was used to predict binding capacity of peptide sequences in the p210 peptide to H-2Db and H-2Kb. The 5 peptides with

the highest predicted binding affinity for H-2Db and H-2Kb according to the artificial neural network binding model *(43–46)* were depicted in the table. There is a strong correlation between binding MHC-I binding affinity and the capacity of peptides to induce a CTL response, with a proposed affinity threshold of 500 nM *(47)*.

Statistical Analysis

Statistical analysis was performed with Graphpad Prism. For comparisons of multiple treatment groups with a control group a one-way ANOVA was performed. For comparisons of data from multiple treatment groups and multiple time points, a two-way ANOVA was used. The Holm-Šídák posttest was used to correct for multiple comparisons. An unpaired two-tailed T test was used for pairwise comparisons. A p-value < 0.05 was considered statistically significant.

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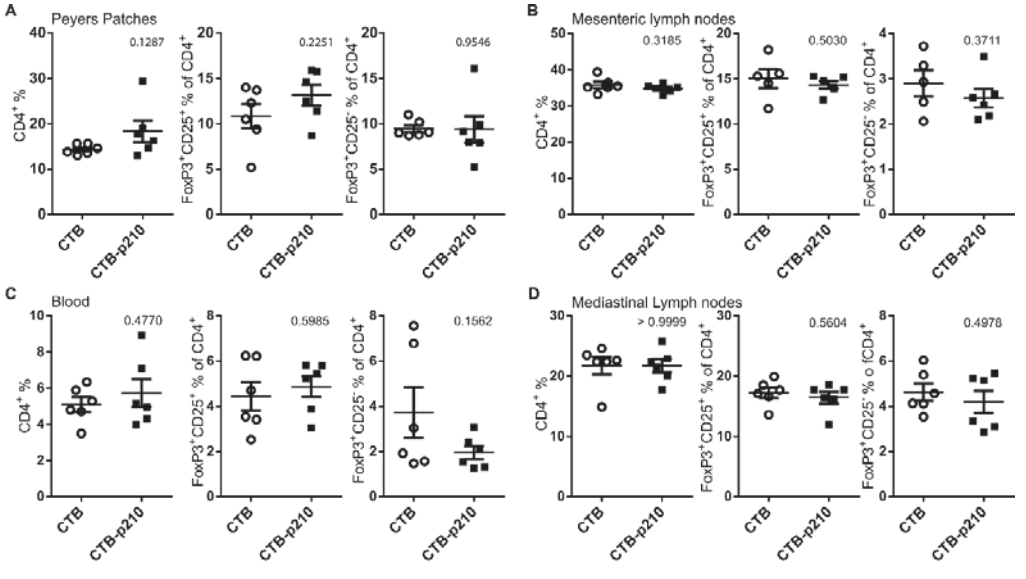
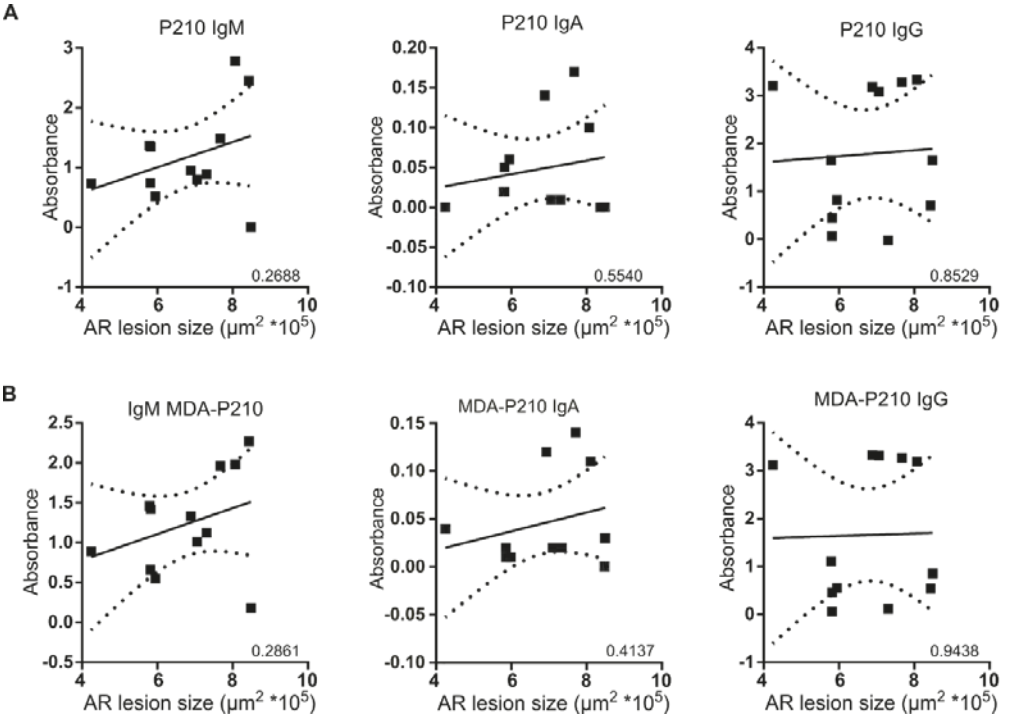
Supplementary figures



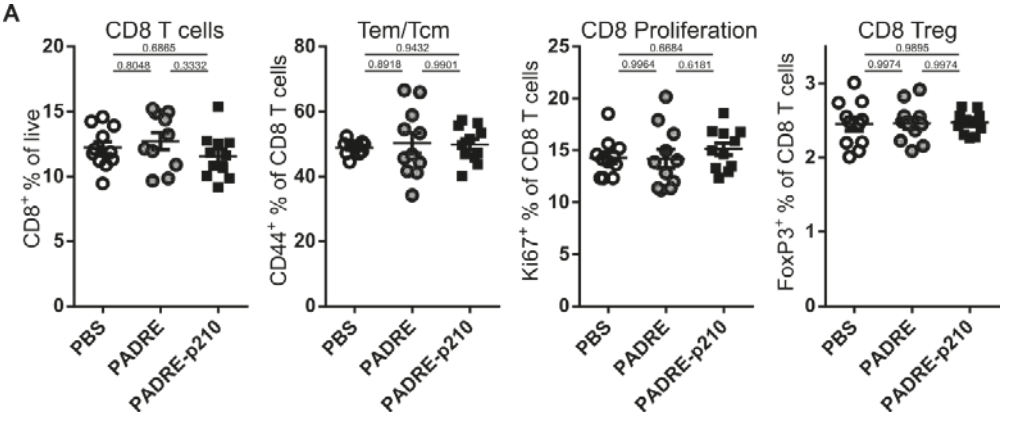
Supplementary Fig. S1 ***Oral CTB-p210 administration does not affect CD4 Treg levels.*** *Quantification of CD4 T cell levels, and FoxP3⁺CD25⁺ and FoxP3⁺CD25⁻ cell content of the CD4 T cell population based on flow cytometry measurements, of* ***A****) Peyers patches,* ***B****) mesenteric lymph nodes,* ***C****) the blood, and* ***D****) mediastinal lymph nodes. Represented as mean ± SEM, unpaired two-tailed T test.*



Supplementary Fig. S2 ***Anti-p210 antibody levels do not correlate with lesion size in CTB-p210 treated HuBL mice.*** *Correlation analysis of the average atherosclerotic lesion size in the aortic root and* ***A****) anti-p210 antibody levels and* ***B****) anti-MDA modified p210 antibody levels in blood serum from CTB-p210 treated animals obtainded after sacrifice. The trend line (solid line), 95% confidence interval (dotted lines), and p value (bottom right) are depicted in the scatter plots.*

Supplementary Table 1 **Best predicted H-2Kb and H-2Db binding peptides in p210**

Allele	Sequence	IC50 (ANN)	Allele	Sequence	IC50 (ANN)
H-2Kb	QSFDLSVKA	3986.06	H-2Db	TTKQSFDLSV	32470.27
H-2Kb	TKQSFDLSV	5484.41	H-2Db	TTKQSFDL	34542.56
H-2Kb	TTKQSFDLSV	9584.8	H-2Db	KTTKQSFDLSV	40485.33
H-2Kb	KTTKQSFDL	9731.83	H-2Db	KQSFDLSVKA	41053.05
H-2Kb	KTTKQSFDLSV	14409.83	H-2Db	KTTKQSFDLS	42345.55



Supplementary Fig. S3 ***Alum adjuvanted vaccination with PADRE-p210 does not change splenic CD8 T cell populations. A****) Splenic overall CD8 T cell levels, antigen experienced CD8 T cells (CD44⁺), proliferating CD8 T cells (Ki67⁺), and regulatory CD8 T cell populations (FoxP3⁺) were quantified by flow cytometric analysis. Represented as mean ± SEM, One-Way ANOVA with Holm-Šídák posttest, p values*